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Original Article

Effects of nutritional supplementation with L-arginine on repair of injuries due to muscle strain: experimental study on rats[☆]



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ABSTRACT

Objective: To evaluate the influence of oral supplementation with arginine on regeneration of injuries due to straining of the anterior tibial muscle of rats.

Methods: Twenty-four Wistar rats of weight 492.5 ± 50.45 g were used. Injuries were induced through straining the anterior tibial muscles. The rats were separated into three groups of eight rats each. In the untreated group (UTG), after induction of injuries, the rats were observed for 24 h. In the simulation group (SG) and the arginine group (AG) respectively, the rats received isotonic saline solution and arginine solution via direct gavage, over a seven-day period. At the end of the period, blood samples were collected for serum evaluations of creatine kinase (CK), lactic dehydrogenase (LDH), aspartate aminotransferase (AST) and C-reactive protein (CRP). The right and left anterior tibial muscles were resected for histopathological evaluations on the muscle injuries, investigating edema, hemorrhage and disorganization or morphometric alteration of the muscle fibers. The tissue repair was investigated in terms of proliferation of adipose tissue, angiogenesis and collagen fibers. The ANOVA and Student's t methods were used and $p \leq 0.05$ was taken to be statistically significant.

Results: In the serum evaluations, the AG showed lower CK assay values and higher AST values. In the histopathological evaluation, the UTG presented edema and hemorrhage compatible with injuries due to strain; the SG presented edema and hemorrhage with proliferation of adipose tissue and collagen fibers; and the AG presented not only the findings of the SG but also, especially, intense angiogenesis.

Conclusion: Oral supplementation with arginine did not cause any significant metabolic alterations that would contraindicate its use and it induced angiogenesis during the repair of muscles injured due to strain.

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Efeitos da suplementação nutricional com L-arginina no reparo de lesões por estiramento muscular. Estudo experimental em ratos

R E S U M O

Palavras-chave:
Músculos/lesão
Arginina
Regeneração
Ratos

Objetivo: Avaliar a influência da suplementação oral com arginina na regeneração de lesão por estiramento do músculo tibial anterior de ratos.

Método: Usaram-se 24 ratos Wistar ($492,5 \pm 50,45$ gramas), induzidos com lesão por estiramento dos músculos tibiais anteriores e separados em três grupos com oito ratos cada. No grupo não tratado (GNT), após a indução das lesões, os ratos foram observados por 24 horas, nos grupos simulação (GS) e arginina (GA) receberam, por gavagem diariamente, respectivamente solução salina isotônica e solução de arginina, durante sete dias. Ao término dos períodos foram coletadas amostras de sangue para as avaliações séricas de creatina-quinase (CK), desidrogenase láctica (LDH), aspartato-aminotransferase (AST) e proteína C reativa (PCR). Foram ressecados os músculos tibiais anteriores (direitos e esquerdos) para avaliações histopatológicas das lesões musculares e pesquisa de edema, hemorragia, desorganização ou alteração morfométrica das fibras musculares. E foi feita a reparação tecidual, para pesquisa da proliferação de tecido adiposo, angiogênese e fibras colágenas. Empregaram-se os testes ANOVA e t de Student com $p \leq 0,05$ para significação estatística.

Resultados: Nas avaliações séricas o GA mostrou valores menores nas dosagens de CPK e maiores nas dosagens de AST. Nas avaliações histopatológicas, no GNT foram evidenciados edema e hemorragia compatíveis com lesões por estiramento, no GS edema, hemorragia com proliferação de tecido adiposo e fibras colágenas e no GA. Além dos achados do GS destacou-se intensa angiogênese.

Conclusão: A suplementação oral com arginina não causou alterações metabólicas importantes que contraindiquem seu uso e induziu angiogênese durante a reparação de lesões musculares por estiramento.

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Introduction

Physical activity is one of the ways of delaying the development of chronic non-transmissible diseases and an increasingly large number of studies have contributed new knowledge on the acute and chronic effects of physical exercise, thereby demonstrating the benefits to health that come from exercise.¹ In the light of this evidence, along with the spread of gyms and sports centers and new possibilities for practicing sports, occurrences of different forms of trauma through excessive demands for muscle strength have also increased, especially due to many bad practices or practices that are not guided by professionals within this field.^{2,3} Muscle trauma accounts for high numbers of injuries in professional and recreational sports and may occur through various mechanisms. Consequently, there has been a proportional increase in the number of studies relating not only to the process of muscle regeneration, but also to new therapeutic options for the various injuries that affect the musculoskeletal system.⁴ Immobilization is generally the method of choice for treating these injuries, although this has the implication of structural alterations such as atrophy, proliferation of connective tissue, fibrosis, loss of muscle extensibility and resistance, and also metabolic disorders.^{5,6} The therapeutic methods used include combinations of immobilization, low temperature at the site, compression and elevation, ultrasound and laser

rays.^{7,8} Revascularization is a determining factor for regeneration of the muscle fiber after injury,⁹ since this enables access to nutrients and oxygenation from vessels in the adjacent tissues, which is fundamental to tissue repair.¹⁰ This revascularization occurs by means of proliferation of endothelial cells, stimulated by means of growth factors such as basic fibroblastic growth factor (bFGF) and vascular endothelial growth factor.¹¹ Arginine is a basic amino acid and is a precursor for synthesizing nitric oxide, which is a molecule of great biological importance, among other molecules.¹² Traditionally, this has been considered to be a non-essential amino acid for adults and children because of the organism's capacity to synthesize it.¹³ However, under certain stressful conditions, its consumption increases such that this exceeds the capacity for endogenous production of arginine. In such situations arginine becomes a conditionally essential amino acid.¹⁴ Nitric acid is involved in a large variety of biological functions.¹⁵ It functions as a vasoactive regulator, promotes endothelial relaxation with consequent vasodilatation, and thus increases the blood flow to the injured tissues.¹⁶ It also performs an important role in the immunological response through mediating cytotoxicity and nonspecific defense mechanisms in the host.¹⁷ Currently, muscle injuries form a group of disorders that are among the most challenging within sports traumatology, especially among athletes who are considered to be at a high-performance level. Although muscle injuries are common, controversy still surrounds their treatment, which is

often inefficient. It is common for athletes with such injuries to require long periods away from their activities before they can fully return to them, and in some cases sequelae may form part of the final result.¹⁸ In this light, the present study had the objective of evaluating the influence of oral supplementation with L-arginine on regeneration of muscle injuries due to strain, induced in the anterior tibial muscles of rats.

Materials and methods

This study was approved by the ethics committee for experimental research (report no. 023/12), in accordance with the protocol of the institution at which it was conducted. Twenty-four adult rats of Wistar lineage were used (*Rattus norvegicus, albinus*), of mean weight 492.5 ± 50.45 g, originating from the vivarium of the Federal University of Paraná (UFPR). They were kept in a specific environment with automatically controlled temperature ($20 \pm 4^\circ\text{C}$) and humidity, and with light and dark cycles of 12 h each. They received specific feed (Nuvilab®, Quimidia S/A) and water ad libitum.

Study design

The rats were subjected to traction to perform passive straining of the anterior tibial muscle of the right hind leg, for 45 min. The left hind leg was kept intact as a control. Following this, the rats were divided into three groups of eight rats each. Twenty-four hours after traction period, the untreated group (UTG) underwent cardiac puncture under anesthesia in order to collect blood in a volume sufficient to induce cardiorespiratory arrest. This blood was subsequently used for biochemical evaluations. After death had been verified, the right and left anterior muscles were resected for histopathological evaluations. The simulation group (SG) underwent daily oral treatment with an isotonic saline solution for seven days. The arginine group (AG) underwent daily oral treatment with an arginine solution in doses of 3 g, diluted in isotonic saline solution, for seven days. On the seventh day after the procedure to induce injuries through muscle strain, the rats in the SG and AG underwent the same procedures as in the UTG, under anesthesia.

The rats were anesthetized both to induce muscle injury through straining and to collect blood. For this purpose, the rats were first sedated through inhalation of halothane (Tanohalo®; Cristália) in a closed circuit and then weighed on an electronic scale (Coleman®). Following this, they were anesthetized using an association of 100 mg/kg of ketamine (Ketamin®; Cristália) and 10 mg/kg of xylazine (Calmium®; Agner União) intraperitoneally, which ensures anesthesia for a minimum of 4 h.

Induction of muscle injury

It was standardized that traction would only be performed on the right hind leg. The apparatus used had previously been described¹⁹ and had been specially constructed for the purpose of inducing muscle injury through straining, as demonstrated in Fig. 1. In this, groups of five rats under anesthesia were individually fixed in dorsal decubitus to the

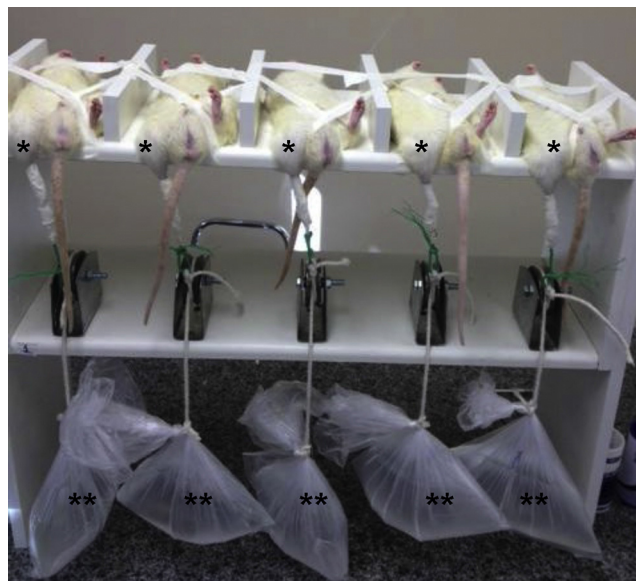


Fig. 1 – Details of the system used for inducing strain in the anterior tibial muscles of rats under anesthesia: (*) right hind legs under traction; () pulleys enabling suspension of plastic bags containing water.**

apparatus using adhesive tape, with the dorsum of the paw of the right hind leg attached to a string using adhesive tape. This string passed over a pulley and a freely hanging plastic bag containing a volume of water corresponding to 150% of the weight of the respective rat was attached to the other end of the string. In this manner, plantar flexion was performed for 45 min, which caused an injury due to straining of the anterior tibial muscle. After induction of the muscle injury, the rats were transferred to a specific environment. The UTG rats were kept there for 24 h and the SG and AG rats for seven days. After these maintenance periods and the nutritional supplementation described earlier, blood collection was performed, followed by resection of the anterior tibial muscles of both of the hind legs, for serum biochemical and histopathological evaluations.

Supplementation with arginine

An amount of 420 g of L-arginine (Merck®) was used, diluted with isotonic saline solution sufficiently to yield 112 ml, which was then sterilized by means of filtration through MF membranes (SCWP304F0 Millipore®). To administer this, the rats were sedated by means of inhalation of halothane (Tanohalo®, Cristália) in a closed circuit. Following this, 0.8 ml of the solution (which contained 3 g of L-arginine) was administered once a day for seven days, always at the same time of day.²⁰

Biochemical evaluations

The serum levels of the following were determined: creatine kinase (CK; U/L), lactic dehydrogenase (LDH; U/L) and aspartate aminotransferase (AST; U/L), by means of an automated enzymatic photometric method; and C-reactive protein (CRP; mg/dl) by means of ultrasensitive turbidimetric immunoassay.

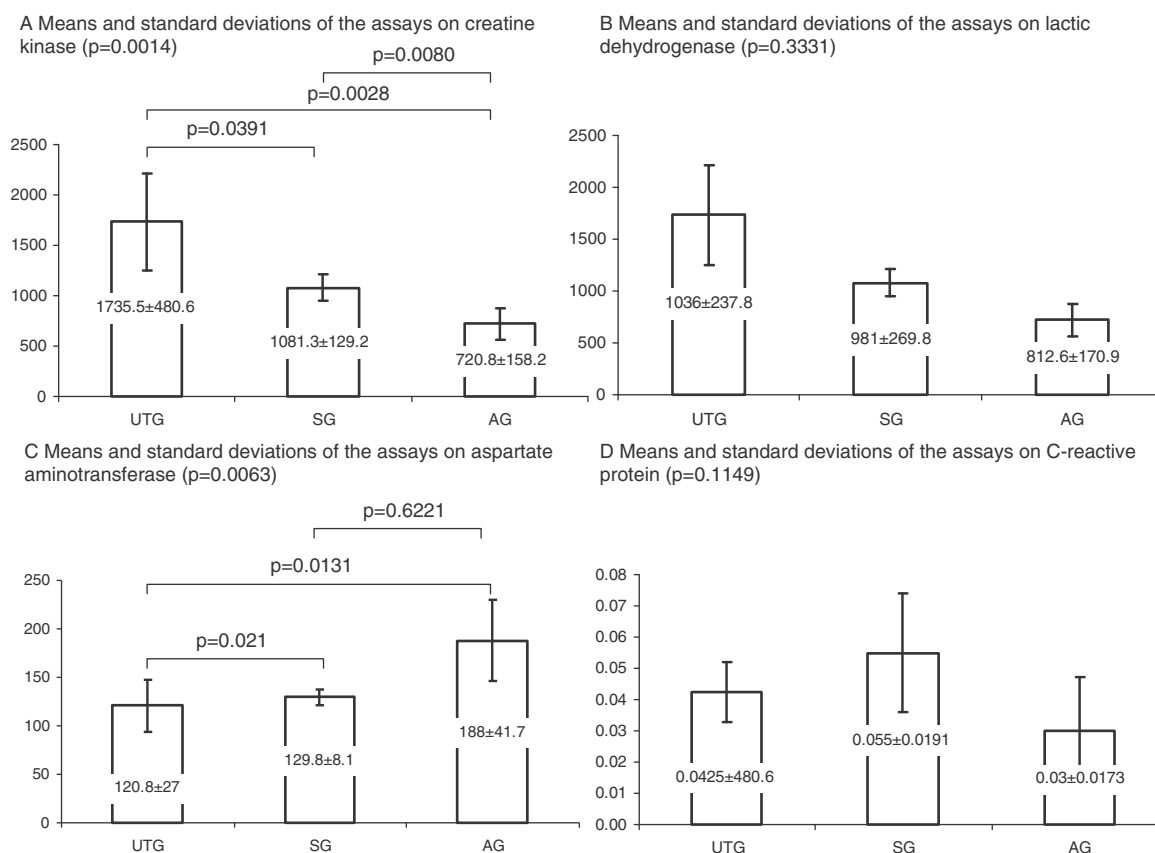


Fig. 2 – Graphs representing the means and standard deviations of the biochemical assays, comparing the three groups: UTG (untreated and subjected to muscle traction), SG (simulation) and AG (arginine).

Histological evaluations

The histological processing was standardized for all the samples from the anterior tibial muscle and began with fixing the muscles in formalin, followed by cutting of standardized sections in planes transversal and longitudinal to the muscle fibers. Automated histological processing was then performed, followed by Harris hematoxylin and eosin staining. The histological slides were analyzed under an optical microscope and were described histopathologically at magnifications of 50, 100 and 400 by two pathologists independently, with emphasis on findings compatible with lesions due to strain, such as edema, hemorrhage and morphometric disorganization or alteration of the muscle fibers. To show the tissue repair, histological patterns consisting of proliferation of adipose tissue, angiogenesis and muscle fibers were sought. The samples from the anterior tibial muscles of the left hind leg, which had not been subjected to traction, were taken to be controls.²¹

Statistical evaluations

The ANOVA and Student *t* tests were used, and the value of 0.05 was taken to define statistical significance among the variables evaluated.

Results

Biochemical evaluations

In the biochemical evaluations, there was a difference between the groups with regard to the means from the assays on creatine kinase ($p=0.0014$) and aspartate aminotransferase ($p=0.0150$). Regarding the means from the assays on lactic dehydrogenase ($p=0.3331$) and C-reactive protein ($p=0.1149$), there was no difference between the groups (Fig. 2). It can be seen from detail A of Fig. 2 that the mean from the assay on creatine kinase in the UTG was significant greater than the means in the SG and AG, and that the mean in the AG was significantly lower than the mean in the SG. In detail C, it can be seen that there was a significant difference between UTG and SG, but not between SG and AG.

Histological evaluations

No histopathological alterations were shown by any of the samples from the left-side hind legs. In evaluating the muscles of the right limbs, which underwent traction as described in Fig. 3, the UTG samples showed tearing of the muscle fibers, edema and hemorrhage among the muscle fibers, morphometric alterations such as tortuous muscle fibers, and

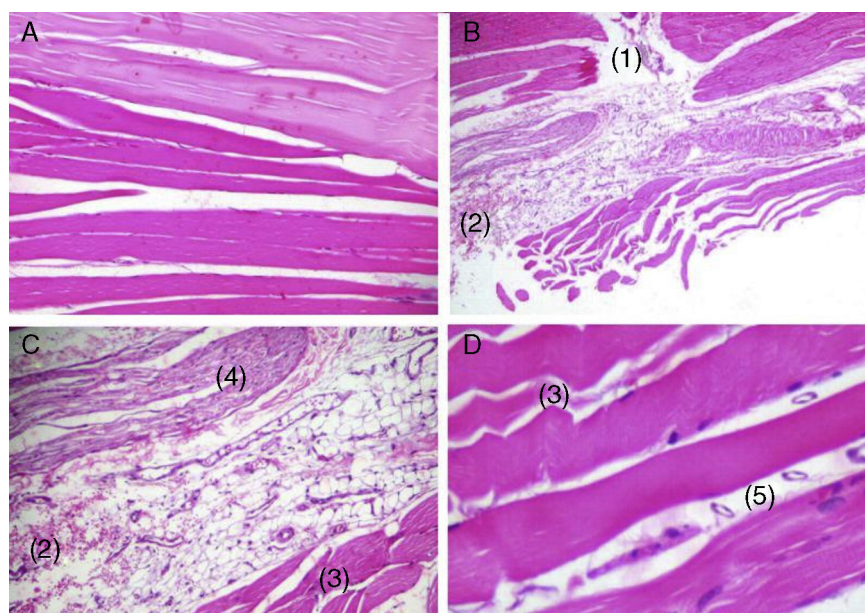


Fig. 3 – Photomicrographs of histological sections through striated skeletal muscle tissue of the samples from the anterior tibial muscle of rats, stained with Harris hematoxylin and eosin Harris. Detail [A]: longitudinal section ($\times 40$) through non-tractioned control muscle, without alterations. Detail [B]: longitudinal section ($\times 40$) through tractioned muscle from untreated rat (UTG), showing tearing of muscle fibers (1), edema and extravasation of red blood cells (hemorrhage) among the muscle fibers (2). Detail [C]: longitudinal section ($\times 40$) through tractioned muscle from untreated rat (UTG), showing edema and extravasation of red blood cells (hemorrhage) among the muscle fibers (2), tortuous muscle fibers (3) and hypereosinophilic fibers with loss of striations, characterizing hyaline degeneration (4). Detail [D]: longitudinal section ($\times 100$) through tractioned muscle from rat treated with arginine (AG), with evidence of intense angiogenesis among the muscle fibers (5), edema and tortuous fibers.

hypereosinophilia with loss of striations, which characterized hyaline degeneration. In the samples from the tractioned muscles from the rats treated with arginine (AG), intense angiogenesis between the muscle fibers could be seen, along with edema and tortuous fibers.

Discussion

Experimental studies contribute toward elucidating the main aspects of the muscle regeneration process because of the high degree of morphological similarity of musculoskeletal tissues among mammals, on the signs of injury and muscle regeneration.²² In the present study, the samples from the anterior tibial muscles of the rats in the UTG were evaluated early on, i.e. 24 h after the injury had been induced, on order to establish the nature of the histological pattern compatible with muscle injury due to strain, as shown by the occurrences of edema and hemorrhage in all the samples. This confirmed the effectiveness of the method chosen for the induction. Different experimental methods for inducing muscle injury have been used, including bruising,²³ electrostimulation,²⁴ physical exercise,²⁵ injections of myotoxins²⁴ and denervation.²⁶ In the present study, it was decided to use a strain model in which histopathological alterations similar to those observed in humans would be induced.¹⁹ The doses of anesthetic used during induction of the lesions were calculated such that these would keep the animals under anesthesia for 4 h and would

avoid use of anti-inflammatory drugs or sedatives that might interfere with the muscle repair process. Regarding the histological results from this study, the UTG presented histological findings compatible with injuries due to strain, as described in details B and C of Fig. 3, in which edema and hemorrhage are shown among the muscle fibers. The vasodilatation promoted by nitric oxide after metabolism of L-arginine has been seen to result in increased muscle perfusion and diminished glucose consumption by the skeletal muscles, thus causing diminished muscle fatigue and inducing improvement of physical performance.²⁷ A dose of 3 g of arginine administered orally was indicated in a study on humans that demonstrated improved resistance to fatigue upon great effort²¹ and in another that showed increased strength and muscle mass in individuals undergoing a weight training program.²⁸ In the present study, there were no intercurrents during the oral treatment with arginine. From Fig. 2, it can be seen that there was a significant decrease in serum creatine kinase levels in the rats that were treated. This enzyme has been used as an indicator of the stress imposed on the skeletal musculature, resulting from intense physical activity, and also as a factor for monitoring the training load.²⁹ In the UTG, the mean creatine kinase level was 1375 ± 480.6 U/L, which was significantly greater than the means in the SG and AG. This group not only had the function of demonstrating the effectiveness of the model used,¹⁹ but also showed consequent increases in the circulating creatine kinase levels. It needs to be borne in mind that the assays on this enzyme in this group were carried

out 24 h after the muscle strain was halted, while the assays on the SG and AG were carried out seven days afterwards. A significant difference ($p = 0.0080$) was observed between these groups, which shows that L-arginine may have a reparative effect with regard to the stress imposed on the anterior tibial muscle through the provocative traction. Thus, arginine may be a potential treatment for these lesions, given that intense angiogenesis was observed in the histological evaluations on the rats that were treated with 3 g of arginine orally for seven days. This effect was already known from muscle training with weights, in which administration of arginine provides greater resistance and gains in mass, and also contributes toward diminishing the percentage of body fat.²⁸ In evaluating the aspartate aminotransferase levels, it was observed that there was a significant increase in the serum levels in the rats of the AG (Fig. 2). An increase in the concentration of cytosolic proteins in the circulation after exercise reflects muscle injury. The proteins evaluated in these situations are frequently creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase and myoglobin, which are usually incapable of crossing the plasma membrane.³⁰ The metabolic processes relating top physical activity are improved through using arginine, since this provides improvements in blood perfusion in muscles and thus induces greater release of nutrients. In this manner, the muscles become capable of producing energy for longer times, along with oxygen, which retards the anaerobiosis of the process. It also favors elimination of toxic substances that accumulate while physical activity is being practiced and makes muscle recovery easier.²⁸

Conclusion

Use of arginine administered orally did not cause significant metabolic alterations that would contraindicate its use and it induced angiogenesis during the repair of muscles injured through strain.

Conflicts of interest

The authors declare no conflicts of interest.

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